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Research Note

Adherence of Eosinophils to the Epicuticle of Infective Juveniles of *Anisakis simplex* (Nematoda: Anisakidae)

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ABSTRACT: We previously have observed that eosinophils were prominent in chronic granulomas around third-stage juveniles of *Anisakis simplex* surgically implanted in laboratory mice and destruction of the juveniles within the host tissues was initiated by day 14 postinfection (PI). Based on histology, the mechanism underlying destruction of these juveniles appeared to involve the interaction of eosinophils on the helminth's cuticle. To test this hypothesis, eosinophils were harvested by bronchoalveolar lavage from CBA/J mice previously infected with a closely related ascaridoid and added to cultures of juvenile worms in the presence or absence of sera from *A. simplex*-immune mice. Adherence of eosinophils occurred only in the presence of *A. simplex*-immune serum taken 14 or 21 days PI from mice infected with 10 worms. Transmission electron microscopy revealed active degranulation of eosinophils onto the epicuticle of the parasite. Destruction of the cuticular surface of the juveniles by eosinophils was not evident after 24 hr of in vitro culture. Apparently, eosinophils lack the ability to kill the juveniles. Damage to the cuticle, however, was observed in areas associated with macrophage adherence.

KEY WORDS: parasitic nematode, *Anisakis simplex, Toxocara canis,* CBA/J mice, eosinophil adherence, epicuticle, juvenile–eosinophil interactions, antibody.

Eosinophils represent a prominent cellular component of the inflammatory response surrounding tissue-invading helminths and have been demonstrated in vivo to adhere to infective juvenile parasites. Linkage of eosinophils to infective juveniles leads to degranulation of the toxic contents of the cells and often results in damage to the surface or killing of the parasites (James and Colley, 1978; Kazura and Grove, 1978; Ramalho-Pinto et al., 1978; McLaren and Ramalho-Pinto, 1979; Kazura and Aikawa, 1980; Desakorn et al., 1987).

One ascaridoid parasite, *Toxocara canis,* the canine roundworm, apparently can avoid the deleterious effects of the immune-mediated adherence of eosinophils. Badley et al. (1987) suggested that infective juveniles of this nematode avoided the toxic effects of eosinophils by rapidly sloughing areas of its epicuticle in contact with the granulocyte; thus, the juveniles continually were releasing surface antigens. This method of evading the host's immune attack might explain the ability of juvenile *T. canis* to migrate with relative impunity through the visceral organs of the body. In contrast, Jones et al. (1990) showed that juvenile *Anisakis simplex* did not survive in vivo beyond 3 weeks postinfection (PI). Using SEM and TEM, we analyzed the ability of eosinophils to adhere to the epicuticle of *A. simplex.*

Third-stage juveniles (L_3) of *A. simplex* were removed from the viscera of several species of Pacific rockfishes (*Sebastes* spp.) collected from the Pacific Northwest in 1989. L_3 were isolated from fish viscera by the pepsin-hydrochloric acid process (Deardorff and Throm, 1988), rinsed several times in sterile PBS, and maintained at ambient temperature in RPMI-1640 tissue culture medium (Sigma Chemical Co., St. Louis, Missouri) containing 50 μg gentamicin sulfate/ml (Sigma).

Eggs, isolated from mature *T. canis,* were cultured in 0.1 N H_2SO_4 for 28 days in the dark at room temperature to allow for embryonation. Following microscopic verification that eggs contained infective second-stage juveniles, the cultures were stored at 4°C until needed. Three mice were placed under mild ether anesthesia and infected with 250 eggs by gastric intubation. These procedures are described in detail by Kayes (1984) and Kayes et al. (1986).

Female CBA/J mice were selected for our experiments because previous reports demonstrated this species is a good experimental model for human anisakiasis (Jones et al., 1990) and provides an excellent source of eosinophils (Kayes et al., 1987). CBA/J mice, obtained from the

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Jackson Laboratories (Bar Harbor, Maine), were maintained on standard rodent Chow and water ad libitum in the Animal Health and Resources facility of the College of Medicine, University of South Alabama. These facilities meet the guidelines established by the National Institutes of Health in the *Guide for the Care and Use of Laboratory Animals.* All mice were obtained as weanlings (weight, 12–15 g) and allowed a minimum of 7 days to acclimate to these facilities prior to inclusion into the experiments.

L_3 of *A. simplex* were rinsed in sterile RPMI-1640 without antibiotics prior to placement into the abdominal cavity of the mice. Mice were anesthetized with ether vapor; a surgical incision was made over the lateral peritoneal cavity; 2, 5, or 10 viable and undamaged L_3 were placed in the abdominal cavity; and the wound was closed with 9-mm stainless steel wound clips. Surgical implantation was selected over gavage techniques as the method of infection because of the size of the mice in relation to the large L_3. Mice were necropsied at days 14 and 21 PI and sera were collected and pooled for each group of mice. Antibody titers for the immune mouse sera (IMS) and normal mouse serum (NMS) were determined using an ELISA assay specific to IgG- and IgM-*A. simplex* excretory–secretory products (ASEX) following the methods adopted by Kayes et al. (1985).

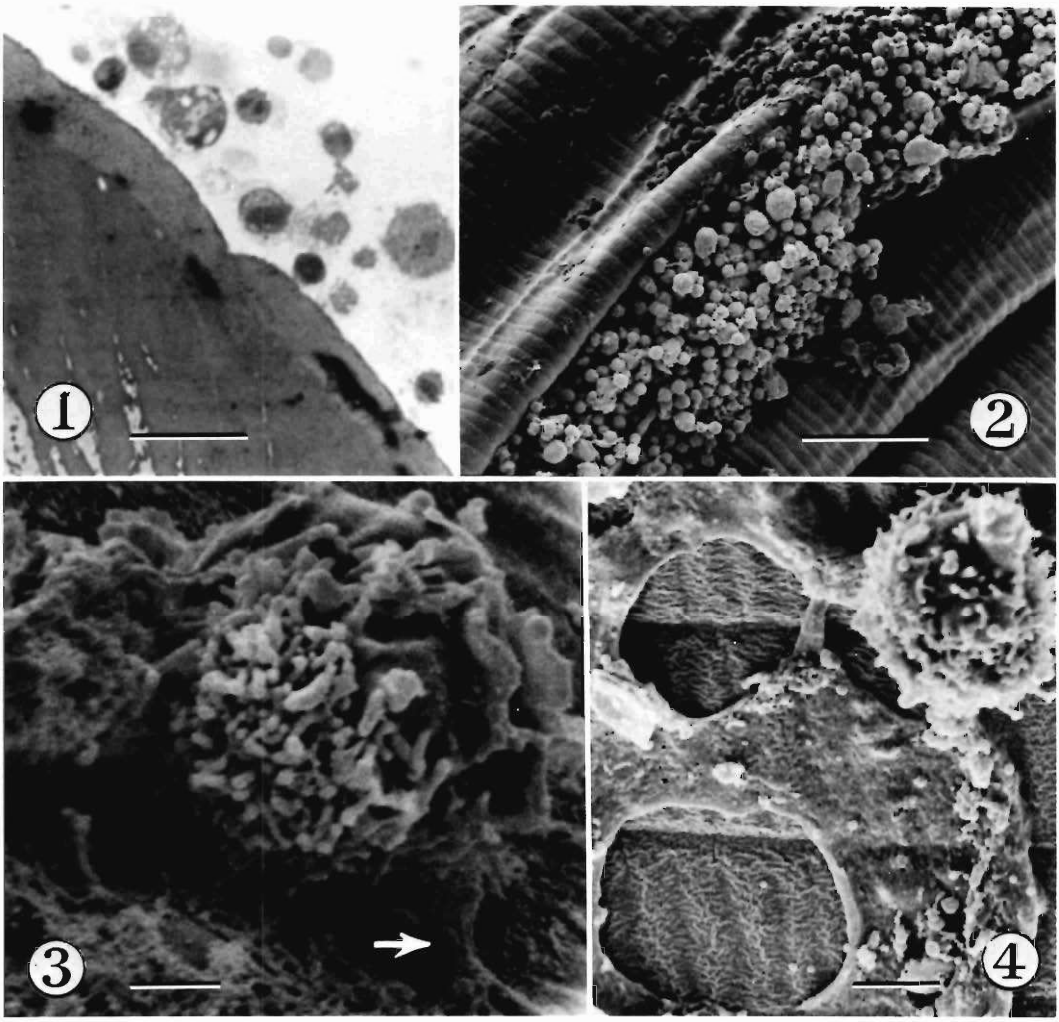
For removal of complement, the procedure of Capron et al. (1981) was followed. Briefly, 100 mM EDTA was added to tubes from each group and the tubes were incubated at 37°C. After 1 hr, sera were removed, dialyzed against Dulbecco balanced saline solution (DBSS; GIBCO, Long Island, New York) for 2 hr at 4°C, and subsequently dialyzed twice against 0.85% NaCl for 2 and 12 hr, respectively.

On day 14 PI, eosinophils were isolated from the lungs of infected mice following the procedures for bronchoalveolar lavage (BAL) as outlined by Kayes et al. (1986, 1987) with slight modification. Briefly, mice were exsanguinated under ether vapor, the thoracic cavity was opened, and the trachea exposed. A MINISET® 21-gauge, 1.9-cm needle with 9-cm-long tubing (Baxter Laboratories, Inc., McGaw Park, Illinois) was inserted into the trachea and a silk ligature was secured around both the trachea and needle. A 1-cc syringe containing 0.5 ml of DBSS with 60 U of heparin/ml was attached to the secured needle, and the contents were infused into the lungs and immediately aspirated back into the syringe. The syringe was detached from the needle and the infusate collected in a 5-ml sterile culture tube maintained at 0–2°C. The process was repeated until the culture tube was full. Collected infusate was centrifuged at 400 *g* for 10 min and resuspended in RPMI-1640. The BAL procedure was selected over peritoneal lavage because it yields relatively pure populations of eosinophils and does not need to be elicited with chronic irritants such as proteose peptone.

An aliquot of 9.0×10^6 cells collected by BAL was treated as described by James and Colley (1978). Briefly, the eosinophils were exposed to a 5-mg/ml solution of bovine pancreatic trypsin (Type 1, 2X crystallized, Sigma) in DBSS, pH 7.6. Cells were suspended at a ratio of 0.1 ml of packed cells to 2 ml of enzyme solution and were incubated at 37°C for 45 min. Soybean trypsin inhibitor (Type 1-S, Sigma), mixed at 15 mg of inhibitor to 5 mg trypsin, terminated the trypsinization process. Eosinophils were washed 3 times in RPMI-1640 prior to use. Three viable juvenile *A. simplex* were introduced into separate 16-mm-diameter tissue culture wells (Costar, Cambridge, Massachusetts) containing 1.5×10^6 cells in 300 μl of RPMI-1640. Each well then received an additional 200 μl of IMS, NMS, or RPMI-1640. Three additional culture wells were set up with similar components as mentioned above but with frozen worms substituted for viable worms.

Alternatively, cytophilic antibodies were removed from a second aliquot of 9.0×10^6 cells by incubation in DBSS, pH 4, for 1 min and washed 3 times in RPMI-1640. Viable worms were exposed to identical conditions as group 1. All cultures were placed in a 37°C incubator with 5% CO_2 in air for 24 hr; after incubation they were removed and fixed.

Worms were fixed directly in the culture wells with cold 0.1 M phosphate-buffered 3% (v/v) glutaraldehyde and refrigerated for 24 hr. Evidence of cellular adherence to *A. simplex* was observed using an inverted microscope. Specimens for scanning electron microscopy (SEM) were dehydrated, critical point-dried in liquid carbon dioxide, mounted on a specimen stub, coated with gold palladium, and examined with a Philips 501 scanning electron microscope. For transmission electron microscopy (TEM), L_3 with attached eosinophils were cut with a razor blade into small pieces, postfixed in 1% osmium te-



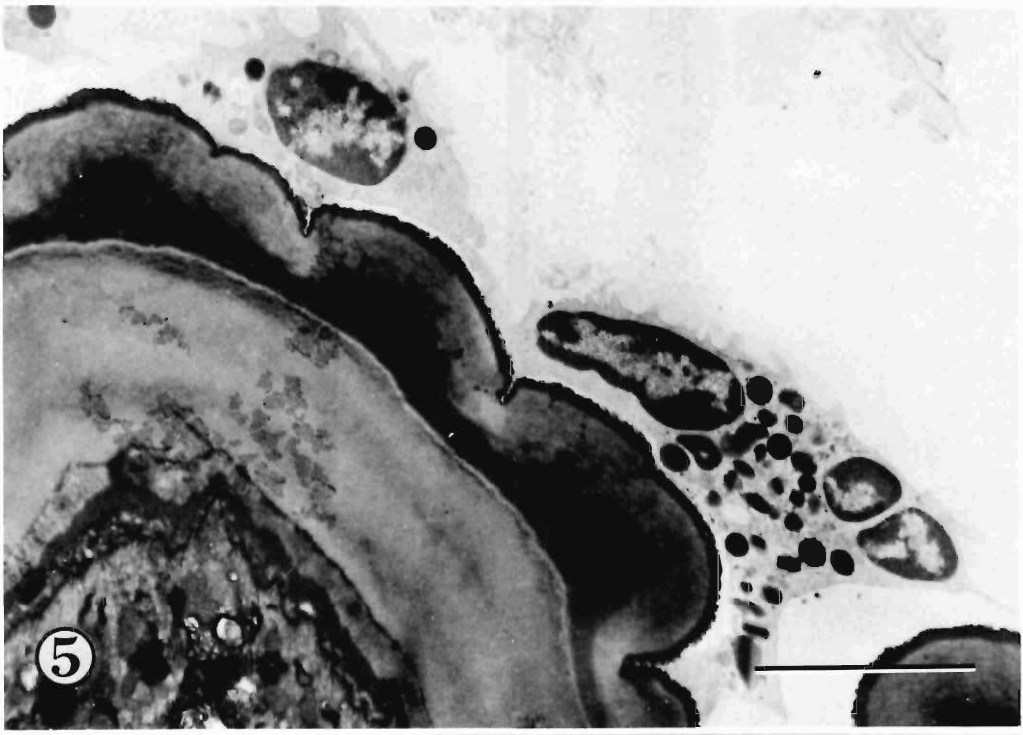
Figures 1–4. 1–3. Adherence of eosinophils on epicuticle of *Anisakis simplex* third-stage juvenile. 1. A cross section of worm showing stratification of cells on cuticle. Bar represents 20 μm. 2. Scanning electron photomicrograph showing aggregation of cells. Bar represents 50 μm. 3. Scanning electron photomicrograph of an eosinophil showing extrusion of pseudopod (arrow). Bar represents 2 μm. 4. Scanning electron photomicrograph showing normal surface topography in areas where eosinophils were removed. Cuticle on right shows normal cuticle without eosinophil contact. Bar represents 4 μm.

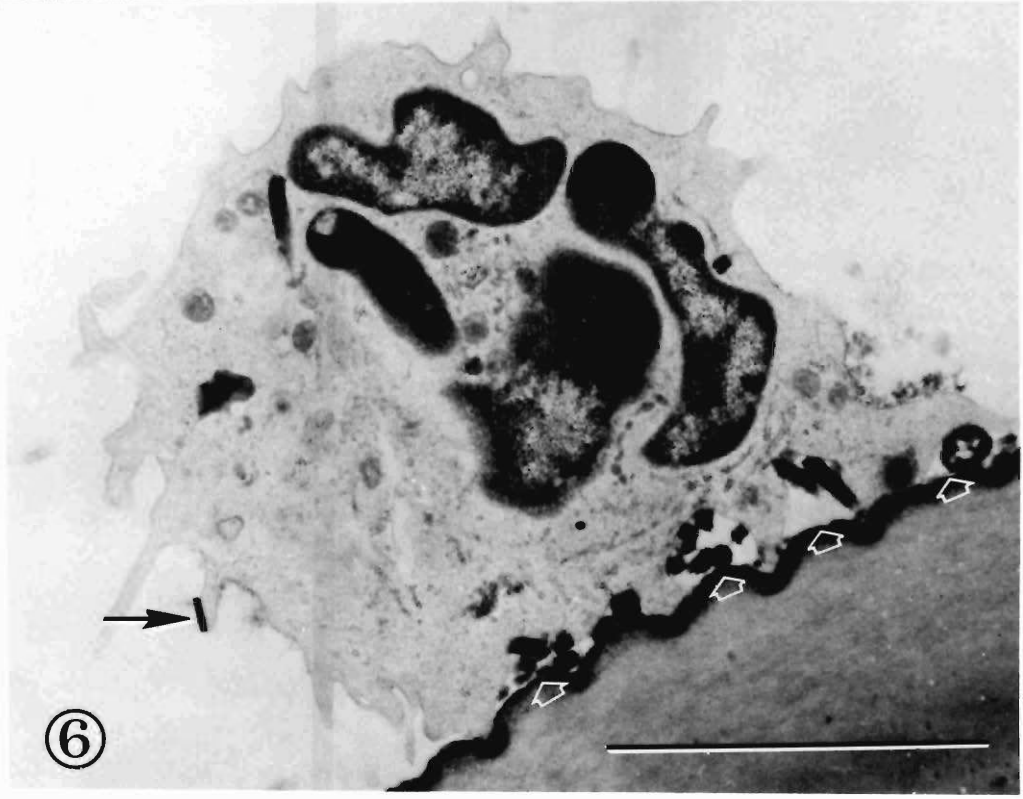
troxide for 2 hr, dehydrated in ethanol, treated with acetone, and embedded in Spurr's low viscosity resin. Thin sections were cut with a diamond knife, mounted on uncoated copper grids, stained with uranyl acetate and lead citrate, and examined with a Philips 301 electron microscope.

For light microscopic analysis, 1-μm thick orientation sections were mounted on glass slides and stained with toluidine blue.

Cells recovered by BAL were approximately 95% eosinophils, as determined by light microscopic observation of stained smears. Macrophages represented a small portion of the remaining 5% of the cell population. These findings were subsequently confirmed by SEM and TEM examination.

Eosinophils attached to the surface of the L_3 in the presence of *A. simplex*-immune serum regardless of the method used to remove the cytophilic antibodies (Figs. 1–3). Eosinophil attachment was predominantly on 1 side of viable





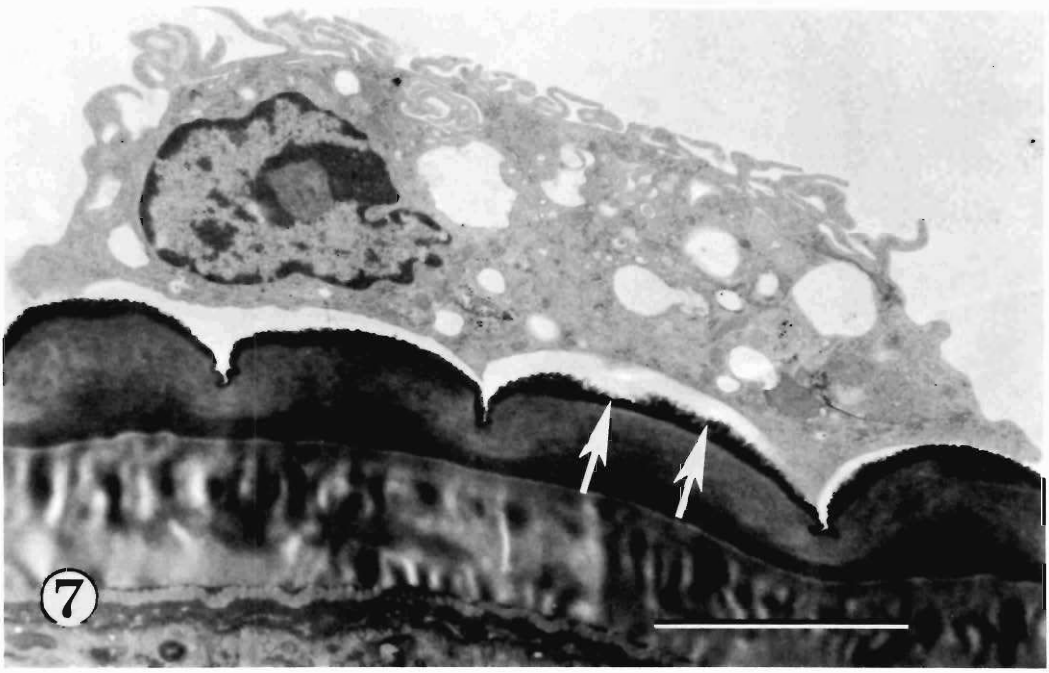


Figure 7. Cross section of juvenile *Anisakis simplex* showing intimate contact with macrophage and areas of cuticular alteration (arrows). Bar represents 6 μm.

worms with the preponderance of the cells affixed near the anterior extremity of the L_3. The vigorous motility of these worms in culture, as viewed through an inverted microscope, removed attached cells on the surface. Adherence was reduced in cultures with NMS and lacking in cultures deficient in IMS. Cells generally attached to the epicuticle in large aggregations, often several layers thick (Figs. 1, 2). No damage to the surface could be seen in areas where cells had been attached but were no longer present (Fig. 4). TEM revealed attached eosinophils and macrophages strictly conforming to the surface topography of the juveniles (Fig. 5). Following attachment, eosinophil granules were seen in vacuoles of plasma membranes juxtaposed to the epicuticle (Fig. 6). Some cuticular damage was evident at the site of macrophage attachment (Fig. 7).

The anterior extremity of the L_3, where large numbers of eosinophils were attached, corresponds with the areas where ASEX are released. Our findings are consistent with those of Sakanari et al. (1988) who showed that the excretory pore and opening of the dorsal gland of the esophagus were involved in the in vivo release of ASEX in humans.

Even though we observed no damage to the cuticle attributable to eosinophils and immune serum, we did notice cuticular alterations associated with the attachment of antibody-sensitized macrophages. This suggest that these cells could be involved in the killing of the juveniles. Several researchers have observed damage or cytocidal events to parasites mediated by macrophages (Haque et al., 1980; Ouaissi et al., 1981; Yen et al., 1986a, b).

Eosinophils that were recovered from *T. canis*-

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Figures 5, 6. TEM micrographs of eosinophils attached to epicuticle of juvenile *Anisakis simplex*. 5. Cellular adherence closely conforming to the annulated contours of cuticle. Bar represents 5 μm. 6. Eosinophil in process of degranulation. Hollow arrows indicate eosinophil granules being released into extracellular pockets and solid arrow shows crystalloid core apparently being released at tip of pseudopod. Bar represents 7 μm.

infected mice and used in adherence experiments with *A. simplex* without first removing cytophilic anti-*T. canis* antibodies were minimally adherent. They displayed specificity for their anisakid targets only after their surfaces were stripped of cytophilic antibodies and reconstituted with anti-*A. simplex* antibodies. Whether the same antibodies that mediate eosinophil adherence also mediate eosinophil cytotoxicity remains unknown.

Sera from mice infected with *T. canis* mediate adherence of eosinophils to the surface of second-stage juveniles of *T. canis* and the adsorption of these sera with homologous excretory–secretory (ES) products completely abolished this ability (Badley et al., 1987). Even though electron microscopy showed these cells to be tightly adherent to the parasite surface, they were unable to mediate a cytotoxic reaction against these juveniles. Because a diffusely granular material was formed on the surface of the epicuticle, it was postulated that the release of ES-like products prevented the killing of juveniles.

We observed no comparable granular product on the worm surface. This observation is peculiar in light of our previous finding that surgically implanted juveniles are rapidly killed (Jones et al., 1990) and our present electron microscopic images which indicate essentially no damage to the epicuticle after eosinophil degranulation on the surface of juvenile *A. simplex*. We conclude, therefore, that the eosinophil is not the major assassin of juvenile ascaridoids as seen with infections of juvenile schistosomes (Ramalho-Pinto et al., 1978; McLaren and Ramalho-Pinto, 1979; Vadas et al., 1980) and *Trichinella spiralis* (Kazura and Grove, 1978; Kazura and Aikawa, 1980; Butterworth and Richardson, 1985).

In conclusion, we determined that eosinophils attach to and degranulate on juveniles of *A. simplex,* IMS was required to induce maximum attachment, and the epicuticle lacked damage at the site of eosinophil adherence. These reports are similar to those of Badley et al. (1987) for *T. canis*. Our findings, however, principally differ from Badley et al. (1987) because juveniles of *A. simplex* did not detach areas of their epicuticle that were in contact with the eosinophils, even though our worms were exposed to the cells 47 times longer than theirs. *Toxocara canis* apparently has evolved this cuticular-sloughing phenomenon to avoid the host's immune response. Thus, in naturally occurring infections in humans or experimental infections in our mouse model, juveniles of *T. canis* can complete their complex tissue migration with relative impunity. In contrast, juveniles of *A. simplex,* whether infecting humans or CBA/J mice, do not survive. This may be the result of their inability to slough their cuticle and the cytotoxic effects associated with the adherence of macrophages.

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Literature Cited

Badley, J. E., R. B. Grieve, J. H. Rockey, and L. T. Glickman. 1987. Immune-mediated adherence of eosinophils to *Toxocara canis* infective larvae: the role of excretory-secretory antigens. Parasite Immunology 9:133–143.

Butterworth, A. E., and B. A. Richardson. 1985. Factors affecting the levels of antibody- and complement-dependent eosinophil-mediated damage to schistosomula of *Schistosoma mansoni* in vitro. Parasite Immunology 7:119–131.

Capron, M., H. Bazin, M. Joseph, and A. Capron. 1981. Evidence for IgE-dependent cytotoxicity by rat eosinophils. Journal of Immunology 126:1764–1768.

Deardorff, T. L., and R. Throm. 1988. Commercial blast-freezing of third-stage *Anisakis simplex* larvae encapsulated in salmon and rockfish. Journal of Parasitology 74:600–603.

Desakorn, V., R. Suntharasamai, S. Pukrittayakamee, S. Migasena, and D. Bunnag. 1987. Adherence of human eosinophils to infective filariform larvae of *Necator americanus* in vitro. Southeast Asian Journal of Tropical Medicine and Public Health 18:66–72.

Haque, A., M. A. Ouaissa, M. Capron, and A. Capron. 1980. Macrophage cytotoxicity by IgE against microfilaria of *Dipetalonema viteae* in vitro. Clinical and Experimental Immunology 40:487–495.

James, S. L., and D. G. Colley. 1978. Eosinophil-mediated destruction of *Schistosoma mansoni* eggs in vitro. II. The role of cytophilic antibody. Cellular Immunology 38:35–47.

Jones, R. E., T. L. Deardorff, and S. G. Kayes. 1990. *Anisakis simplex*: histopathological changes in experimentally infected CBA/J mice. Experimental Parasitology 70:305–313.

Kayes, S. G. 1984. Spleen cell responses in experimental murine toxocariasis. Journal of Parasitology 70:522--529.

———, **R. E. Jones, and P. E. Omholt.** 1986. Pul-

monary granuloma formation in murine toxocariasis: transfer of granulomatous hypersensitivity using bronchoalveolar lavage cells. Journal of Parasitology 74:950–956.

———, ———, **and** ———. 1987. Use of bronchoalveolar lavage to compare local pulmonary immunity with the systemic immune response of *Toxocara canis*-infected mice. Infection and Immunity 55:2132–2136.

———, **P. E. Omholt, and R. B. Grieve.** 1985. Immune responses of CBA/J mice to graded infections with *Toxocara canis* infective larvae. Infection and Immunity 48:697–703.

Kazura, J. W., and M. Aikawa. 1980. Host defense mechanisms against *Trichinella spiralis* infection in the mouse; eosinophil mediated destruction of new-born larvae in vitro. Journal of Immunology 124:355–361.

———, **and D. I. Grove.** 1978. Stage-specific antibody dependent eosinophil-mediated destruction of *Trichinella spiralis*. Nature 274:355–361.

McLaren, D. J., and F. J. Ramalho-Pinto. 1979. Eosinophil-mediated killing of schistosomula of *Schistosoma mansoni* in vitro: synergistic effect of antibody and complement. Journal of Immunology 123:1431–1438.

Ouaissi, M. A., A. Haque, and A. Capron. 1981. *Dipetalonema viteae*. Ultrastructural study of the in vitro interaction between rat macrophages and microfilariae in the presence of IgE antibody. Parasitology 82:55–68.

Ramalho-Pinto, F. J., D. J. McLaren, and S. R. Smithers. 1978. Complement-mediated killing of schistosomula of *Schistosoma mansoni* by rat eosinophils in vitro. Journal of Experimental Medicine 147:147–156.

Sakanari, J. A., H. M. Loinaz, T. L. Deardorff, R. B. Raybourne, J. H. McKerrow, and J. G. Frierson. 1988. Intestinal anisakiasis: a case diagnosed by morphologic and immunologic methods. American Journal of Clinical Pathology 90:107–113.

Vadas, M. A., A. E. Butterworth, B. Sherry, A. Dessein, M. Hogan, D. Bout, and J. R. David. 1980. Interactions between human eosinophils and schistosomula of *Schistosoma mansoni*. I. Stable and irreversible antibody-dependent adherence. Journal of Immunology 124:1441–1448.

Yen, P. K. F., P. G. Holt, N. F. Stanley, J. M. Papadimitriou, and T. Robertson. 1986a. In vitro antibody-mediated macrophage activity on *Breinlia macropi* microfilariae. II. Ultrastructural and video recording investigations of adherence and cytotoxicity. Parasite Immunology 8:201–216.

———, ———, ———, **and K. J. Turner.** 1986b. In vitro antibody-mediated macrophage activity on *Breinlia macropi* microfilariae. I. Adherence and cytotoxicity. Parasite Immunology 8:139–147.

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Research Note

Sphyranura euryceae (Monogenea) on *Eurycea* spp. (Amphibia: Caudata), from Northcentral Arkansas

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ABSTRACT: *Sphyranura euryceae* Hughes and Moore, 1943 (Polystomatoidea: Sphyranuridae) was found on the external gills, skin, and gular folds of 10/10 larval cave salamanders, *Eurycea lucifuga,* and 10/10 neotenic graybelly salamanders, *E. multiplicata griseogaster,* in northcentral Arkansas. This represents the second time *S. euryceae* has been reported and constitutes new host and distributional records for the parasite. A summary is presented on the *Sphyranura* spp. from caudate amphibians. Based on morphological similarities and the opinions of earlier workers, the synonymy of *S. polyorchis* Alvey, 1936, with *S. osleri* Wright, 1879, is provisionally supported.

KEY WORDS: cave salamander, *Eurycea lucifuga, E. multiplicata griseogaster,* graybelly salamander, Monogenea, Polystomatoidea, prevalence, *Sphyranura euryceae, S. oligorchis, S. osleri, S. polyorchis,* Sphyranuridae, synonymy.

Wright (1879) described *Sphyranura osleri* from the gills and mouth cavity of mudpuppies, *Necturus lateralis* (syn. of *N. maculosus*). Wright and Macallum (1887) provided additional information about the species. Since then, additional *Sphyranura* spp. have been described or reported from North American hosts (Alvey, 1933a, b, 1936; Hughes and Moore, 1943; Coggins and Sajdak, 1982). The purpose of this note is to report *Sphyranura euryceae* Hughes and